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Video Article

Reprogramming Primary Amniotic Fluid and Membrane Cells to Pluripotency in Xeno-free Conditions

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Abstract

Autologous cell-based therapies got a step closer to reality with the introduction of induced pluripotent stem cells. Fetal stem cells, such as amniotic fluid and membrane mesenchymal stem cells, represent a unique type of undifferentiated cells with promise in tissue engineering and for reprogramming into iPSC for future pediatric interventions and stem cell banking. The protocol presented here describes an optimized procedure for extracting and culturing primary amniotic fluid and membrane mesenchymal stem cells and generating episomal induced pluripotent stem cells from these cells in fully chemically defined culture conditions utilizing human recombinant vitronectin and the E8 medium. Characterization of the new lines by applying stringent methods – flow cytometry, confocal imaging, teratoma formation and transcriptional profiling – is also described. The newly generated lines express markers of embryonic stem cells – Oct3/4A, Nanog, Sox2, TRA-1-60, TRA-1-81, SSEA-4 – while being negative for the SSEA-1 marker. The stem cell lines form teratomas in scid-beige mice in 6-8 weeks and the teratomas contain tissues representative of all three germ layers. Transcriptional profiling of the lines by submitting global expression microarray data to a bioinformatic pluripotency assessment algorithm deemed all lines pluripotent and therefore, this approach is an attractive alternative to animal testing. The new iPSC lines can readily be used in downstream experiments involving the optimization of differentiation and tissue engineering.

Video Link

The video component of this article can be found at https://www.jove.com/video/56003/

Introduction

The technology of induced pluripotent stem cells (iPSC) brings about potential cell replacement therapies, disease and developmental modeling, and drug and toxicological screening[1,2,3]. Replacement therapies can conceptually be achieved by cell injection, in-vitro differentiated tissue (such as cardiac patches) implantation, or guided regeneration by means of tissue engineering. Amniotic fluid (AFSC) and membrane stem cells (AMSC) are an excellent source of cells for these interventions either directly[4,5,6,7] or as a starting cell population for reprogramming into pluripotency[8,9,10,11,12].

Early approaches used undefined culture systems or reprogramming methods that require entail genomic integration of constructs[9,10,11,12]. A more recent study employed a xeno-free medium, even though a less defined basement membrane attachment matrix (BMM) was used, to generate iPSC from amniotic fluid epithelial cells. However, the teratoma formation assay was not included in the study along with a wealth of in-vitro and molecular data. Amniotic fluid epithelial cells were found to have a roughly 8-fold higher reprogramming efficiency when compared to neonatal fibroblasts[13]. In another study, mesenchymal stem cells from amniotic fluid were also found to be reprogrammed into iPSC with a much higher efficiency[12].

Pluripotent stem cells can be differentiated into tissues representative of all 3 germ layers and thus have the broadest potential. Pediatric patients could benefit from the harvesting, reprogramming, and tissue engineering of their autologous amniotic fluid stem cells prenatally and amniotic membrane stem cells perinatally. Furthermore, the relatively low level of differentiation of fetal stem cells (lower than adult stem cells[14,15]) could theoretically aid in addressing the observed retention of epigenetic bias from source cells in iPSC[16].

Here we present a protocol for reprogramming amniotic fluid and membrane stem cells to pluripotency in chemically defined xeno-free E8 medium on recombinant vitronectin[17] (VTN) using episomal plasmids[18]. The main advantage of amniotic fluid and membrane cells as a source of cells for reprogramming lies in their availability pre- and perinatally and thus this approach would mainly benefit research into pediatric tissue engineering.

Protocol

The protocol follows institutional guidelines of the ethics committee for human research. Written consent of the patient was obtained for using the amniotic fluid for research.

This protocol follows the policies of the Institutional Animal Care and Use Committee of the University of South Alabama.

1. Isolation and Culture of Primary Amniotic Mesenchymal Stem Cells

1. **Plating of amniotic fluid cells**
    1. Obtain a minimum of 2.5 mL of amniotic fluid harvested in the process of amniocentesis by a physician.
    NOTE: All handling of live cells and tissue must be performed in a sterile tissue-culture cabinet and proper personal protective equipment must be used. Familiarity with basic cell culture and sterile technique is required.
    2. Prepare the amniotic fluid and membrane cell (AFMC) culture medium: EBM-2 basal medium, 15% fetal bovine serum (FBS), 20 ng/mL of bFGF, 25 ng/mL of EGF, 10 ng/mL of IGF. For culture of primary amniotic fluid as well as amniotic membrane stem cells, the medium should be supplemented with antibiotic-antimycotic solution.
    3. On day 0, mix 2.5 mL of amniotic fluid with 3.5 mL of AFMC culture medium and plate into a T25 flask. Incubate at 37 °C and 5% CO_2 for at least 48 h undisturbed before checking for the presence of colonies.
    4. On day 5, colonies of adherent cells should be present. Gently rock the flask to dislodge cells that did not fully adhere to the bottom and debris and vacuum-aspirate the spent medium/amniotic fluid mixture using a Pasteur pipette. Replace with 5 mL of fresh AFMC medium.
    5. Culture for another 5 days. Change medium every other day as described in step 1.1.4.

2. **Isolation of primary mesenchymal stem cells from human amnion**
    1. Obtain placentas as soon as possible following birth, within 24 h at the latest, to maximize cellular integrity. Cut a 9 cm^2 segment of the amnion, remove blood clots and wash in a 50 mL centrifuge tube with 30 mL of PBS supplemented with antibiotic-antimycotic solution.
    2. Mince the membranes using a pair of scalpels to fine pieces in a sterile 10 cm tissue culture dish. The digestion of the membranes and the extraction of the cells will be achieved using a tissue dissociation system. Follow the manufacturer's protocol.
    NOTE: the finer the pieces of the tissue after mincing, the higher the cell numbers recovered after digestion.
    3. Transfer the minced membrane tissue mass using the scalpel blades into one tissue dissociation tube and mix with 4.7 mL of RPMI 1640 medium. Mix in the dissociation enzymes (see **Table of Materials**).
    4. Mount the tubes onto the tissue dissociator and run program "h_tumor_01". Incubate the tubes at 37 °C on a rocking platform for 30 min.
    5. Further dilute the suspensions with 35 mL of RPMI 1640 and apply to a 70 µm strainer placed over a 50 mL collection centrifuge tube.
    6. Centrifuge for 5 min at 200 x g at room temperature, discard supernatant, resuspend the pellet in 5 mL of RPMI 1640, count the cells using a hemacytometer, and plate at a density of 10,000 cells/cm^2 into tissue culture-treated vessels with freshly prepared AFMC medium supplemented with antibiotic-antimycotic solution.
    NOTE: In case of incomplete digestion, small pieces of tissue will be present and single cells will be scarce. Spin down again and plate the whole pellet into one T75 flask.

3. **Culture of primary AFSC and AMSC**
    1. Passage colonies of AFSC/AMSC by vacuum-aspirating spent medium using a Pasteur pipette and adding 2 mL of cell detachment enzyme into the flask. Incubate at 37 °C for 5-8 min.
    2. Tap on the flask to help dislodge the cells and mix the suspension with an equal volume of AFMC medium (antibiotic-antimycotic supplement should not be necessary from this point on). Centrifuge at 200 g for 4-5 min. Remove the supernatant using either a glass Pasteur pipette or simply by means of inverting the tube and emptying it into a waste container.
    3. Flick the bottom of the centrifuge tube to break up the pellet into a single cell suspension in the remaining drop of liquid and mix with AFMC medium for plating. Plate into T-flasks at a density between 2,500 and 5,000 cells/cm^2.
    4. Change medium every other day. Do not culture the cell lines beyond passage 6. For reprogramming purposes, use as low a passage as possible.
    5. Prepare frozen stocks of AFSC and AMSC as back-ups using freezing medium. Harvest cultures using a cell detachment enzyme, centrifuge at 200 g for 4 °C for 5 min.
    6. Remove the supernatant using a Pasteur pipette and flick the bottom of the tube to singularize the cells in the pellet. Resuspend in complete freezing medium at a density of 1×10^6/mL and aliquot into cryovials. Store in a freezing container overnight at -80 °C. Then move to liquid nitrogen for long-term storage.

2. Reprogramming into Pluripotency

1. **Obtain the reprogramming plasmids**
    1. Purchase the reprogramming plasmids through a non-profit plasmid repository. A Material Transfer Agreement is needed.
    2. Transform the plasmids into *E. Coli* competent cells and isolate the plasmids using a commercial plasmid extraction kit. Follow the manufacturer's instructions.
    3. Measure the concentration of plasmid DNA using a spectrophotometer. Aim for a high resulting plasmid concentration, ideally around 1 µg/µL to avoid dilution of the sample during transfection.
    4. Measure the concentrations of the individual plasmids using a UV-spectrophotometer and aliquot them individually.

5. Mix together 3 µg, 3 µg, and 2 µg of EN2K, ET2K and M2L plasmids, respectively. This is the reprogramming plasmid solution. The amount of plasmid solution is enough to transfect 1 x 10^6 cells. Prepare several such aliquots.
6. Store all aliquots at -80 °C.

2. **Prepare target culture plates**
   1. Coat one 6-well plate with vitronectin – add 1 mL of vitronectin dilution buffer into each well and mix in 40 µL of VTN stock solution (1 µg/cm^2). Leave at room temperature (RT) or in the incubator at 37 °C for 1 h.
   2. Vacuum-aspirate the solution using a Pasteur pipette and replace with 2 mL of AFMC medium in each well. Store at 37 °C until the cells are to be plated.
      NOTE: Important: The AFMC medium used in this step should not contain any antibiotic or antimycotic solutions.

3. **Harvest cultured primary AFSC/AMSC**
   NOTE: Expand AFSC/AMSC in culture enough to make frozen stocks at a low passage number and dedicate one T-75 flask for reprogramming. Since as few as 100,000 cells are sufficient for an experiment, aim at harvesting around 500,000 cells to compensate for losses and if optimization of transfection parameters or different culture conditions are to be tested.
   1. At the earliest convenience at a low passage, harvest the AFSC/AMSC using cell detachment enzyme mix as described in step 1.3.1 to 1.3.2. After the cells have been centrifuged, proceed to the next step.
   2. Resuspend the pellet in 1 mL of PBS and mix well to wash serum components. Count the cells using a hemacytometer. Adjust the cell density to 100,000/mL of PBS and aliquot into 1.5 mL microcentrifuge tubes. This ensures only a minimal contact time between the cells and the buffer used for transfection.
   3. Place the microcentrifuge tube on top of 5 mL polystyrene tubes (as adaptors, will allow centrifugation in a regular swing rotor) and centrifuge at 200 x g for 4 min at room temperature. Invert the tubes and discard the supernatant into a waste container. Do not use a fixed-angle rotor.
   4. Perform an additional centrifugation step at 200 x g for 3 min at room temperature. This will allow the remaining liquid from the walls of the tube to collect at the bottom. Carefully aspirate all of it using a 200 µL pipette.

4. **Transfection with reprogramming plasmids**
   1. For reprogramming experiments, a transfection system (see **Table of Materials**) will be used to deliver reprogramming plasmids into the cells. Place transfection tips, transfection tubes, resuspension buffer, and electrolytic buffer into the tissue-culture cabinet. The kit reagents are kept at RT until they are opened, then they are stored at 4 °C.
      NOTE: We use the 10 µL version of the kit.
   2. Move the transfection device close so that its tube station can be placed directly into the cabinet. Fill one transfection tube with 3 mL of electrolytic buffer and mount the tube into the station by pushing it all the way inside the slot.
   3. Take the reprogramming plasmid solution aliquots prepared in step 2.1.5 out of -80 °C storage and allow them to thaw at RT in the culture cabinet.
   4. On the transfection device, select the following transfection parameters: 950 V, 40 ms, and 1 pulse.
   5. Resuspend the pellet containing 100,000 cells in 10 µL resuspension buffer. Work quickly from this point on since resuspension buffer is slightly toxic and an increased exposure time results in a noticeably lower cell viability.
   6. Mix in 1/10 of the reprogramming plasmid solution (the solution aliquot was prepared for a total of 1 x 10^6 cells).
   7. Mount a transfection tip onto the transfection pipette.
   8. Aspirate the cell suspension into the transfection tip carefully, avoiding formation of air bubbles. If bubbles are observed, expel the suspension and repeat the aspiration. Air bubbles will impede transfection.
   9. Insert the transfection pipette into the transfection tube and press the "START" button on the screen of the transfection device. Wait for the screen message informing about the success of the transfection and remove the pipette from the tube immediately.
   10. Expel the suspension into 1 well of the target 6-well plate prepared in section 2.2. Mix in the medium from a neighboring well and distribute the suspension equally into both wells (the resulting cell density will be 50,000/well).
   11. Repeat the transfection for all microcentrifuge tubes containing AFSC/AMSC individually. Place the plate into the incubator at 37 °C and 5% CO_2.

5. **Culture of transfected AFSC/AMSC**
   1. Culture the transfected cells for 2-5 days. Then switch to reprogramming medium consisting of E8 supplemented with 100 µM of sodium butyrate on day 3.
      NOTE: Secondary passage can be performed to avoid overgrowth of the source AFSC/AMSC. However, the passaging will disable the option to calculate the reprogramming efficiency correctly if this parameter is of interest.
   2. Change the reprogramming medium every day to every other day for 10 days. Change the medium every day from day 10 on.

6. **Manual picking of fully reprogramed colonies for clonal expansion**
   1. Fully reprogrammed colonies appear around day 14. Allow colonies to expand in size and become compact. They can be manually picked and transferred to fresh plates as early as day 15-16.
   2. 1 h before the picking procedure, coat 24-well plates with 8 µL of VTN in 300 µL of vitronectin dilution buffer per well (1 µg/cm^2) and incubate at RT or 37 °C. Replace the solution with E8 medium without sodium butyrate.
   3. Select colonies of a sufficient size (ideally over 400 µm in diameter) in a sterile culture cabinet. A phase contrast microscope or a stereomicroscope can be used.
   4. For picking, an LCD imaging microscope placed in the cabinet will be used since its monitor eliminates the need for oculars. Sterilize the microscope stage with 70% ethanol.
   5. Using a regular phase-contrast cell culture microscope, select, mark, and note the number of colonies to be picked. This is important to make sure time is not wasted for this process during the actual picking.
   6. Fill a number of PCR tubes that is equal to or greater than the number of colonies to be picked with 30 µL of 0.5 mM ethylenediaminetetraacetic acid (EDTA) in PBS. Colonies will be placed into these tubes for partial dissociation before plating.

7. Plan to pick 5 colonies at a time from the plates using a 10 µL pipette set to 2 µL. Hold the pipette tip at an angle at the colony edge and carefully and gradually scrape the whole colony off the surface. Immediately aspirate the whole colony into the pipette tip and transfer it into one of the prepared PCR tubes with EDTA.
8. Repeat with the remaining 4 colonies. Incubate at RT for 4-6 min.
9. Pipet the suspension up and down gently using a larger pipette tip to break the colony down into smaller clumps. Avoid creating a single cell suspension.
10. Plate the suspension directly into a target well of a 24-well plate prepared in step 2.6.2. Repeat with the remaining colonies.
11. Repeat steps 2.6.6 through 2.6.10 if more than 5 colonies are to be picked but do not pick more than 5 colonies at a time. Incubate at 37 °C and 5% CO_2.

7. **Clonal expansion and maturation of iPSC**
    1. Allow colonies to grow and become compact. 3-6 days are sufficient. Change the culture medium daily. Use between 400 µL and 1 mL of E8 medium based on the cell density.
    2. Wells of the 24-well plate with a sufficient colony density will be expanded into 6-well plates. 1 h before passaging, coat the 6-well plates with VTN (as in step 2.2.1). Then replace the solution in the well with 2 mL of E8 medium per well.
    3. Aspirate the spent medium from the source wells using a 1 mL pipette and replace with 300 µL of 0.5 mM EDTA to wash. Aspirate immediately using the same pipette tip and replace with 300 µL of 0.5 mM EDTA again, then incubate at RT for 5 min. Aspirate all liquid using a 1 mL pipette.
    4. Set the 1 mL pipette to capacity, mount a 1 mL wide-bore tip on it, and aspirate the E8 medium from the target well into the tip. Wash the source iPSC culture off with a stream of the medium.
    5. Transfer the suspension into the target well and pipet up and down several times to break up colonies into clumps of 20-50 cells. Make sure they get distributed evenly in the well by gentle rocking and shaking the plate and incubate at 37 °C and 5% CO_2.
    6. Change the medium daily and passage every 3 to 4 days. Any differentiating colonies can be marked under the phase-contrast microscope and removed using a pipette tip in the culture cabinet. This allows for selective propagation of high-quality pure iPSC culture.
    7. 1 h before passaging, coat the wells of a 6-well plate with VTN as in step 2.2.1. Then replace the solution with 2 ml of E8 medium per well.
    8. Routine passaging is similar to the initial passaging done using 0.5 mM EDTA (steps 2.7.2 to 2.7.5). Replace spent medium in one well of a 6-well plate with 1 mL of EDTA to wash and discard.
    9. Add 1 mL of EDTA and incubate at RT for 5-7 min for partial dissociation. Optimize the incubation time if necessary, making sure a suspension of around 20- to 50-cell clumps is produced. Avoid dissociation into single cells.
    10. Discard the EDTA solution and aspirate 1 mL of the E8 medium using a 1 mL pipette from the target well into a wide-bore pipette tip.
    11. Wash off the source iPSC culture with a stream of the E8 medium repeatedly until a desired portion of it was released from the surface and transfer into the target well. This portion represents the split ratio (*e.g.*, 1/8 of the culture can be transferred for a 1:8 ratio.)
    12. Passage every 3-4 days. Allow iPSC lines to mature by culturing them for at least 15 passages before using them in downstream experiments

3. Characterization and Confirmation of Pluripotency

NOTE: Refer to the supplementary files for details on flow cytometry and confocal microscopy.

1. **Teratoma formation assay**
    1. To determine the capacity of iPSC to differentiate into tissues representative of all three germ layers by teratoma formation assay, follow the institutional policies regarding animal care and use, planning ahead to allow time for filing the appropriate protocol documents. The teratoma formation in mice will take between 6-10 weeks.
    2. Culture 4 wells of a 6-well plate per iPSC line for 4 days. The approximate number of cells injected into one flank of a mouse is 0.5 to 1 x 10^6 cells. Optional: an extra well can be dedicated to determining a representative cell number in a well by means of single cell dissociation with a cell detachment enzyme and counting.
    3. Calculate the volume of the E8/BMM mixture the iPSC clumps will be suspended in: Both flanks of a mouse are injected, each with 150 µL of clump suspension. Three mice are sufficient to test teratoma formation of one iPSC line. Include an extra 150 µL per needle in the resulting volume to compensate for dead volume loss. 1 needle per mouse is used. The total volume is therefore 3 * (150 µL * 2 + 150 µL) = 1350 µL
    4. Partially dissociate the iPSC colonies with EDTA as if they were to be passaged, as described in the steps 2.7.8 to 2.7.10. It is important that the colonies be dissociated into clumps and not separated into single cells.
    5. Wash off the iPSC colonies with 675 µL of E8 medium (half of the calculated volume from step 3.3.3), using a wide-bore tip. Transfer the clump suspension into a 5 mL polystyrene tube. Place on ice.
    6. Combine with 675 µL of BMM. Keep the resulting suspension on ice until the injection.
    7. Anesthetize the mice to immobilize them using isoflurane. This should be performed or guided by skilled personnel of the vivarium.
    8. Vortex the 5 mL polystyrene tube briefly and aspirate the clump suspension (450 µL per mouse) into one insulin syringe equipped with a 22 G needle. Inject 150 µL of the cell suspension subcutaneously. This volume contains approximately 1 x 10^6 cells (see step 3.1.2).
    9. Inject 3 mice per iPSC line. Follow proper animal care practice for all procedures.
    10. Monitor the health of the mice daily.
    11. When the teratomas reach an endpoint diameter of 1.5 to 2 cm, euthanize the mice, explant the teratomas, and store them in formalin solution for tissue fixation for 24 h.
    12. Bring the fixed teratomas to a histology core facility for hematoxylin eosin (H&E) staining. A pathologist will grade the presence of tissues of all three germ layers.
        Note for Karyotyping: Live iPSC cultures should be shipped to specialized cytogenetic laboratories for testing of the integrity of the karyotype. It is recommended that this testing be performed every 5 passages.

2. **Transcriptional profiling**
    1. Culture 2 wells of a 6-well plate for 3-4 days to obtain one RNA sample. Only use high-quality cultures, minor contamination with differentiating cells can be addressed by scraping them using a pipet tip.
    2. Isolate RNA from iPSC cultures using a commercially available kit following the manufacturer's protocol. Ship samples to a specialized genomic core facility.
    3. Obtain the global transcriptional profiles of the iPSC lines using microarrays (see **Table of Materials** for supported choices) or RNA sequencing.
    4. Submit "*.idat" files for bioinformatic assessment of pluripotency through an online interface at the Coriell Institute. Alternatively, submit "*.cel" files for bioinformatic identification of cell type, including pluripotent stem cells, through an online interface at Johns Hopkins University. See **Table of Materials** for details on the types of data the individual bioinformatic assays accept.

Representative Results

Informed written consent was obtained from patients before harvesting amniotic fluid for genetic testing purposes and dedicating a small aliquot of the fluid for research. No consent is required for the use of the amniotic membrane in research as the placenta represents medical waste. Amniotic fluid and membrane stem cells display typical mesenchymal properties, morphologically their cells are spindle-shaped and phase-bright. Upon reprogramming, the cells undergo mesenchymal-to-epithelial (MET) transition and acquire cobblestone-like morphology and spatial organization of the colonies, indicating epithelial properties. This process is initiated as early as 48-72 h following the introduction of reprogramming episomal plasmids. Absence of these colonies by day 5 of reprogramming would indicate failure of the experiment. The cells of the MET colonies proliferate and as a result, the colonies become compact, between day 5 and 14. Compact MET colonies are comprised of cells that are not easily individually discernible (**Figure 1A**). On around day 14, fully reprogrammed colonies appear with cells arranged in a monolayer, carrying prominent, easily discernible nuclei and nucleoli. They are ready to be mechanically isolated and expanded when the colonies reach a suitable size and become compact (**Figure 1B**).

Fully and partially reprogrammed colonies are present in the cultures throughout the entire reprogramming period, though partially reprogrammed colonies do not necessarily acquire full pluripotency. **Figure 2** shows a representative flow cytometric analysis of embryonic stem cell (ESC) marker expression in fully and partially pluripotent colonies and their corresponding morphologies. Full pluripotency is associated with the expression of Oct4, Nanog, Sox2, TRA antigens and SSEA-4, while SSEA-1 expression is negative[19,20,21] (**Figure 2A**). Partially pluripotent cells, however, do not express Nanog and TRA antigens[20] (**Figure 2B**). The expression and localization of ESC markers should be confirmed by immunocytochemical staining and imaged using a wide-field or confocal microscope (**Figure 3**).

A functional confirmation of pluripotency is achieved by demonstrating the ability of the iPSC lines to form teratomas following subcutaneous injection of the cells into scid-beige mice. 6-8 weeks are needed for the teratomas to reach the end-point size. H&E staining of the tissues and examination by a pathologist is then performed to confirm the presence of tissues representative of all three germ layers – endoderm, neuroectoderm and mesoderm (**Figure 4A**). An alternative to animal testing is to analyze the transcriptional signature associated with pluripotency by genomic approaches like cDNA microarrays[22,23]. The proportion of the transcriptional profile that overlaps with one of a pool of well-established iPSC and ESC lines can then be quantified by the online bioinformatic pluripotency evaluation software in the form of a plot of two classifiers – pluripotency and novelty (**Figure 4B**). The higher the pluripotency score, the more the query iPSC line resembles the established lines. A high novelty score, however, could indicate deviations or even chromosomal aberrations, despite a high pluripotency score (such as in teratocarcinoma lines)[22]. All iPSC lines generated by following the protocol presented here have been deemed pluripotent by flow cytometry, imaging, teratoma formation, and transcriptional analysis methods.

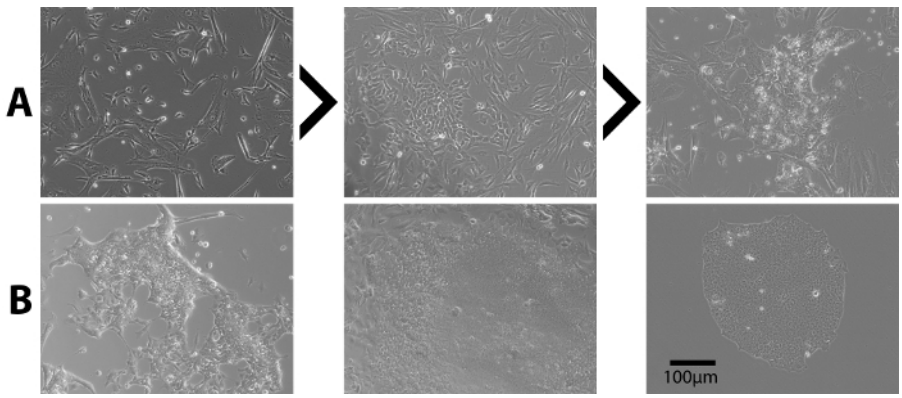

Figure 1: Morphological progression of the cells during reprogramming. (A) The amniotic fluid and membrane stem cells, which represent source cells for reprogramming, display a typical mesenchymal morphology, elongated and phase-bright (left) until they undergo the mesenchymal-to-epithelial transition (MET) which leads to acquisition of epithelial properties and formation of colonies with cobble stone-like cells (center). These colonies proliferate and create irregular cellular masses of MET cells (right). **(B)** At the later stages of reprogramming (starting from around day 14), colonies of fully reprogrammed cells emerge – individually discernible cells with prominent nuclei and nucleoli arranged in monolayers, with well-defined borders (center) – and are present alongside MET colonies that are more numerous (left). A fully reprogrammed isolated mature clone is depicted on the right. Scale bar = 100 µm Please click here to view a larger version of this figure.

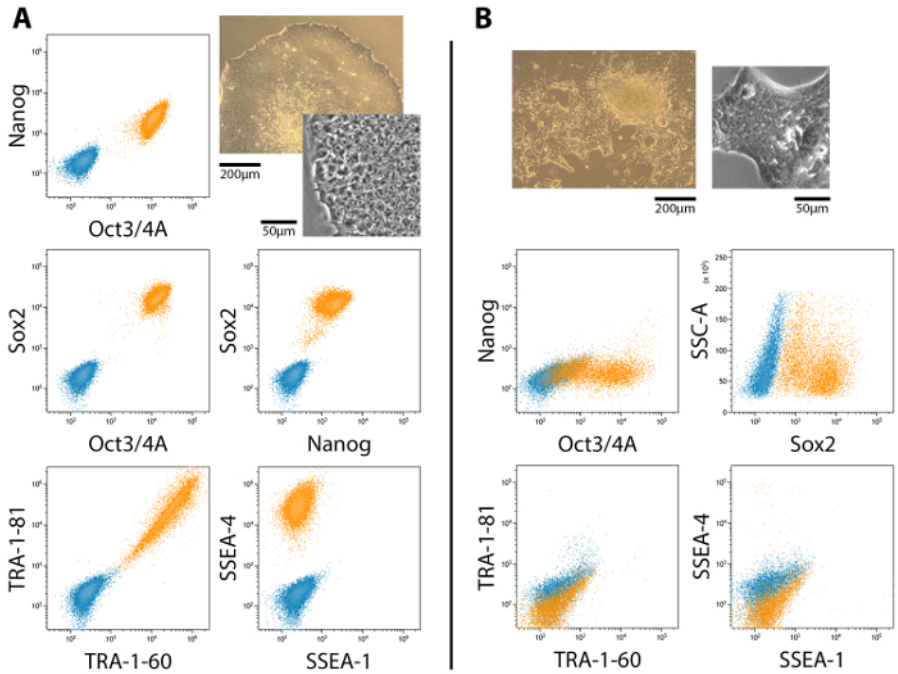

Figure 2: Flow cytometric analysis of the expression of ESC markers in fully and partially (MET) reprogrammed cell colonies. (A) The pluripotent expression profile is positive for Oct4, Nanog, Sox2, TRA-1-60, TRA-1-81 and SSEA-4, while negative for SSEA-1. **(B)** Partially pluripotent cell colonies – those that have undergone the MET but failed to progress to full pluripotency – are positive for Oct4 and Sox2 but Nanog, the TRA and SSEA antigens are absent. The associated morphologies are included for side-by-side comparison. Scale bars = 200 µm and 50 µm. Please click here to view a larger version of this figure.

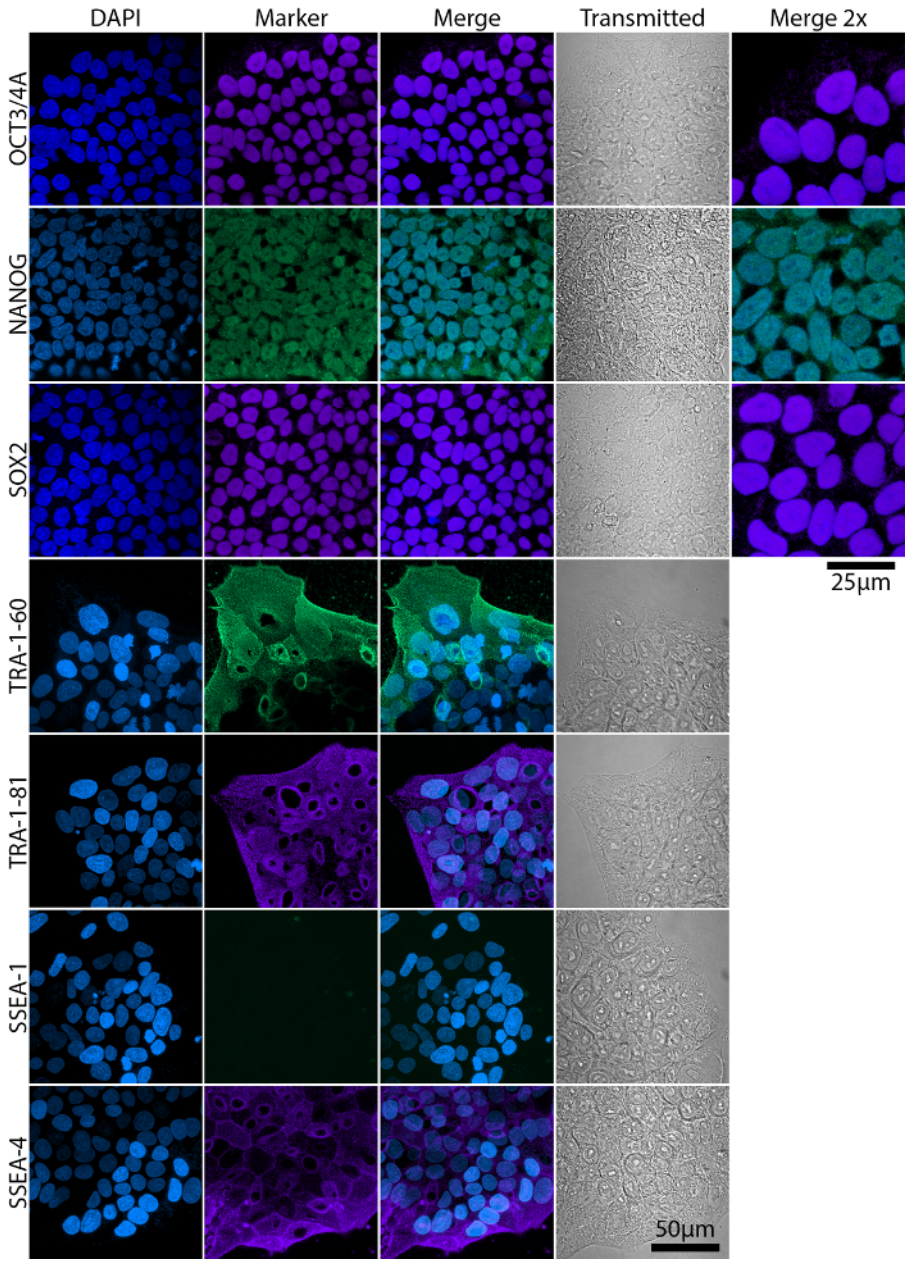

Figure 3: Confocal imaging analysis of the expression of ESC markers in mature amniotic fluid iPSC. Transcription factors Oct3/4A, Nanog and Sox2 are localized in the nuclei while TRA and SSEA antigens are glycoproteins localized on the membrane. Scale bar = 50 µm. Images of greater magnification (Merge 2X) were included for Oct3/4, Nanog and Sox2 for better visualization of their nuclear localization. Scale bar = 25 µm. Transmitted – images acquired on transmitted light. Please click here to view a larger version of this figure.

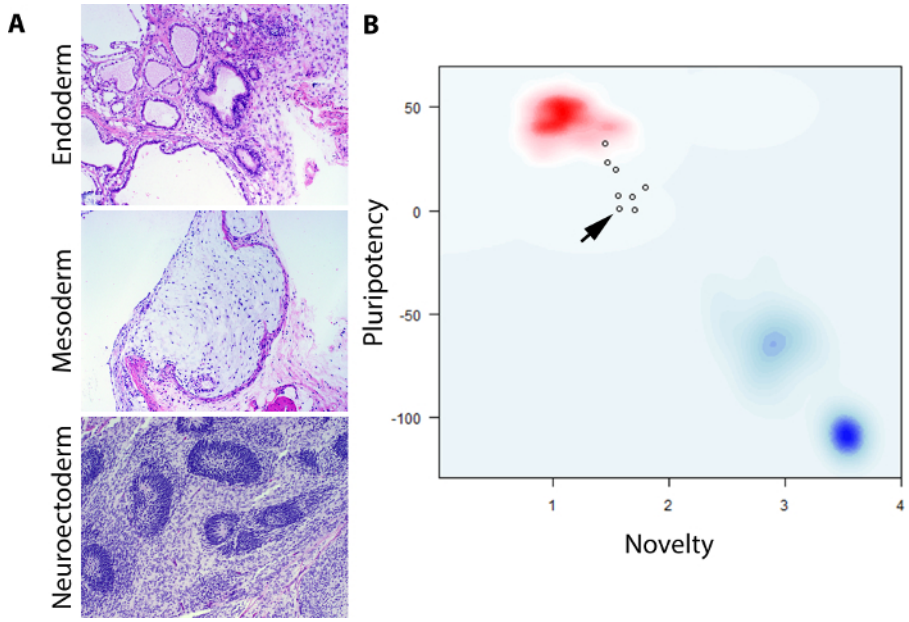

Figure 4: Teratoma formation and transcriptional profiling in mature amniotic fluid and membrane iPSC. (A) Teratomas grown in scid-beige mice subcutaneously contain tissues representative of all three germ layers (100X magnification). **(B)** The global expression microarray profiles submitted to online pluripotency software returned a plot of two classifiers – pluripotency and novelty. High pluripotency scores and low novelty scores - red cloud - indicate an expression profile of a typical ESC/iPSC line. The blue cloud represents a cluster area for differentiated cells, while the faint blue cloud represents a cluster area for partially pluripotent cells. The amniotic fluid (3 lines) and membrane (4 lines) iPSC were deemed pluripotent by the test. An ESC line WA25 was included as a control and is identified here with a black arrow. Please click here to view a larger version of this figure.

Discussion

The initial phase of iPSC generation from fetal stem cells entails the extraction of the source cells from the fetal tissues, their culture, expansion, and introduction of the episomal reprogramming plasmids. This phase is followed by a culture period of around 14-18 days before the first fully reprogrammed colonies can be expanded. The final phase is maturation of the iPSC clones. The initial extraction of amniotic membrane stem cells is achieved by means of a combined mechanical and enzymatic digestion of the amnion. We found that an incubation time of 30 min resulted in the highest number of cells extracted with the highest viability. The digestion procedure can produce small pieces of tissue and cell clumps. If the proportion of these relative to single cells is high, we recommend plating all clumps and single cells into one vessel since all can contribute to outgrowths of adherent cells. Plating amniotic fluid stem cells is straightforward as the cells are only mixed with the culture medium and incubated until colonies of adherent cells reach a sufficient size. Regular tissue culture-treated plasticware is perfectly suitable and we do not recommend specialty surfaces, even though they are intended for improved primary cell culture, since with these we observed lower viabilities and difficulties with the passaging process.

The amniotic fluid and membrane stem cells should be expanded and stocks frozen but, at the earliest convenience, the cells can be used as source cells for reprogramming. For the purpose of the introduction of the episomal plasmids into the cells, the transfection system used here with the transfection parameters set to 950 V, 40 ms, and 1 pulse has performed very well, with all lines attempted ultimately successfully reprogrammed (over 10 lines). The main competing delivery system operating on a similar principle did not produce a successful reprogramming experiment in our hands.

The transfected cells are seeded onto vitronectin-coated dishes in AFMC medium for the first 3-5 days, then the medium is switched to E8 supplemented with 100 µM sodium butyrate. This greatly increases the rate of full pluripotency acquisition. The first signs of morphological transformation can be seen as early as 48-72 h. The source cells undergo the MET and colonies of cells with epithelial morphology appear. These gradually proliferate and become compact. A subset of the colonies will acquire the morphological features of fully pluripotent stem cells – individually discernible cells with prominent nuclei and nucleoli, flat colonies with well-defined borders, as opposed to the fuzzy borders observed in partially pluripotent MET colonies. Upon the moment of the acquisition of full pluripotency, the compact MET cell colonies acquire prominent nuclei and the individual cells become discernible while creating a unique morphological pattern. To a trained eye, this pattern is a clear sign of successful reprogramming. However, to an investigator that lacks PSC culture training, identification of colonies that have successfully progressed to full pluripotency requires careful evaluation as MET and iPSC clones can be mistaken for each other. **Figure 1** and **Figure 2** provide examples of both. If MET clones are picked instead, thorough flow cytometry analysis will reveal the mistake, and in particular, the TRA-1-60 and TRA-1-81 antigens will most likely be absent as shown in **Figure 2**. Indeed, TRA antigens were previously found to be stringent pluripotency markers. However, partially pluripotent MET cells might be of interest in cancer research[25].

This culture condition is suboptimal for the source AFSC/AMSC and eventually, their proliferation will slow down and they will acquire a flatter, fibroblast-like morphology. The source cells form tissues that can detach from the surface during the later stages of reprogramming, though this does not negatively affect the reprogramming process. On the contrary, the process sometimes leads to freeing up space for the reprogrammed

colonies, while eliminating unwanted un-reprogrammed cellular material. Detached tissues can easily be discarded using a sterile pipette tip, leaving partially and fully pluripotent colonies behind, greatly simplifying manual selection downstream.

For manual picking of the fully reprogrammed colonies, we use an LCD imaging system, that can be placed in the safety cabinet, lacking any parts protruding out that would disturb the air flow. Other than this imaging system, no special equipment is needed as the picking itself can be performed using regular pipettes. The picked colonies are partially dissociated in EDTA/PBS solution before being plated into the target wells to grow out as clones. Depending on the line and the clone, for several passages, the cultures may be contaminated with spontaneously differentiating cells. Manual manipulation and serial passaging usually eliminate this problem. Clones riddled with extensive differentiation should be discarded, however, precious clones can be salvaged with various degrees of success by means of repeated manual picking of pluripotent colonies rather than disposing of differentiating cells. Episomal plasmids were shown to take around 15 passages to be lost completely from the iPSC[26]. Therefore, it is advisable to allow the clones to grow for at least that number of passages before using them for downstream applications and analyses, except for routine monitoring of TRA antigen expression and karyotype. TRA antigen expression can easily be monitored by flow cytometry as described here in the protocol, since the assay only requires around 200,000 cells, and can be performed whenever the researchers are in doubt as to whether the cultured clones are maintaining pluripotency properly. Flow cytometry analysis of the ESC marker expression is not considered to be sufficient to confirm pluripotency in candidate lines[19].

Teratoma formation assay is the standard conclusive pluripotency test[27]. PSC grown in chemically defined, xeno-free conditions are particularly susceptible to dissociation-induced death and hence, injecting them subcutaneously as clumps is necessary for their successful implantation[8,28]. Following injection, usually 4-6 weeks are enough for the growth of the xeno-grafts to be visible and before week 8, all can be harvested, H&E-stained and analyzed. Animal welfare, cost, and long testing periods needed are reasons for developing alternative methods. Genomic analyses combined with advanced, machine learning-powered bioinformatic approaches can provide an accurate evaluation of global expression profiles. The cost of obtaining such data is comparable to the cost of the teratoma formation assay, however, the genomic approach is considerably faster and no animals have to be used. One such assay is a bioinformatic pluripotency evaluation software[22]. It is implemented as an online interface (**Table of Materials**). The growing popularity and plummeting cost of RNA sequencing will ensure continuity of this approach. An alternative to this pluripotency software is available from Johns Hopkins University[23] (cellnet.hms.harvard.edu) and is based on a similar approach and is able to accept microarray data to analyze the transcriptome of human samples. The advantage of this software is that it has the ability to identify not only pluripotent stem cells but also differentiated cells and, since its curated datasets were derived from primary tissues, the level of similarity between *in-vitro* grown cells/tissues and *in-vivo* tissues can be determined, providing an excellent quality control for the development of differentiation protocols or tissue engineering. The test has the capacity to classify the queries into 20 different cell or tissue types. At present, it requires microarray data but the authors are working towards expanding the platform options to RNA sequencing as well.

By following the presented protocol, researchers can generate iPSC lines from amniotic fluid and membrane stem cells with a very high reproducibility in fully chemically defined and xeno-free medium and using a non-integrating reprogramming method. These lines can be used in basic research to optimize differentiation protocols and ultimately in disease modeling, drug screening, or pediatric tissue engineering studies.

Disclosures

The authors declare that they have no competing financial interests.

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